

## The involvement of ubiquitin in vegetative desiccation tolerance

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### Abstract

We have isolated a polyubiquitin cDNA from the modified desiccation-tolerant grass *Sporobolus stapfianus*. This cDNA, along with a commercially available polyclonal ubiquitin antibody, was used to characterize desiccation/rehydrated-associated changes in ubiquitin-mediated protein degradation in *S. stapfianus* and the fully desiccation-tolerant moss *Tortula ruralis*. Northern analysis demonstrated that in *S. stapfianus* leaves two ubiquitin transcripts, of ca. 1.4 and 1.2 kb, accumulated above control levels during drying and rehydration but were barely detectable in desiccated tissue. The peak in rehydration-associated transcript accumulation coincided with a depletion in ubiquitin monomer levels indicating an increase in protein degradation. Analysis of *T. ruralis* revealed three ubiquitin transcripts of ca. 1.9, 1.3 and 0.65 kb, with only the 1.3 kb transcript level varying in response to drying and rehydration and all transcripts being stable in dried tissue. Western analysis revealed that conjugated ubiquitin, indicative of proteins targeted for removal, was evident in all samples of *Sporobolus* but detectable only in slow-drying *Tortula* which also displayed reduced levels of ubiquitin monomer. These results demonstrate that desiccated *T. ruralis* gametophyte possesses stable ubiquitin transcripts which can be translated upon rehydration enabling rapid initiation of cellular repair through degradation of certain proteins. This is in contrast to *S. stapfianus* which requires several hours to replenish depleted ubiquitin transcripts. The ubiquitin response to drying and rehydration in evolutionarily diverse systems is characterized, and the role of repair mechanisms such as ubiquitin-mediated protein degradation in desiccation tolerance is assessed.

### Introduction

Most plants can withstand minor fluctuations in water availability through morphological and/or physiological adaptation, but succumb to more extreme conditions. A small number of plants (desiccation-tolerant/resurrection plants) have developed mechanisms allowing them to tolerate drying of their vegetative tissues to air dryness, survive in a dried state for prolonged periods and resume growth upon rehydration (Bewley and Krochko, 1982; Schwab and Gaff, 1990).

Desiccation-tolerant plants are categorized as either fully desiccation-tolerant or modified desiccation-

tolerant. Fully desiccation-tolerant plants are found among the bryophytes, lichens and algae (Oliver and Bewley, 1979; Bewley and Krochko, 1982) and can survive rapid loss of water. Modified desiccation-tolerant plants are found in all the major classes of vascular plants except the gymnosperms (a taxonomic group consisting of the cycads, conifers and gnetophytes) and survive only when drying occurs over several hours or days. For a plant to survive desiccation it is proposed that three criteria have to be met (Bewley, 1979): (1) limitation of damage incurred to a repairable level, (2) maintenance of physiological integrity in the dried state, and (3) mobilization of repair mechanisms upon rehydration. Fully desiccation-tolerant plants, such as the moss *Tortula ruralis*, often experience rapid drying and as a result do not appear to induce major protection mechanisms during

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDJB Nucleotide Sequence Databases under the accession number AF148448.

the initial phases of water loss. Instead, they rely on a rapid implementation of cellular repair mechanisms induced by rehydration, though this appears to be supplemented by a certain level of constitutive cellular protection (Bewley, 1979; Bewley and Krochko, 1982; Bewley and Oliver, 1992; Oliver and Bewley, 1997; Oliver *et al.*, 1997). Modified desiccation-tolerant plants, on the other hand, have time during drying to accumulate putative protective factors such as LEA proteins, including dehydrins (Blomstedt *et al.*, 1988; Close, 1996; Ingram and Bartels, 1996) as well as certain sugars (Kaiser *et al.*, 1985; Bianchi *et al.*, 1992) and amino acids (Bianchi *et al.*, 1992). The slow rate of water loss during the drying event is made possible by morphological and physiological adaptations to retain water in drying atmospheres that these more complex plants have evolved.

Much of the work on desiccation tolerance in plants has focused on modified desiccation-tolerant plants. The drying phase has received particular attention with the identification of numerous dehydration-inducible genes in *Craterostigma plantagineum* (Bartels *et al.*, 1993; Schneider *et al.*, 1993) and *Sporobolus stapfianus* (Blomstedt *et al.*, 1998; O'Mahony and Oliver, 1999). Many of these genes, though not all, are also induced by the phytohormone abscisic acid (ABA) (Furini *et al.*, 1996; Blomstedt *et al.*, 1998) suggesting it plays a role in the signal transduction pathway involved in the induction of at least some portions of the desiccation-tolerance mechanism in these plants. The rehydration phase has received less attention with only a few rehydration-responsive proteins identified (Bernacchia *et al.*, 1996; Oliver *et al.*, 1998; O'Mahony and Oliver, 1999). The rehydration phase is important in modified desiccation-tolerant plants as cellular damage does occur at this time, possibly due to cellular swelling (Bewley, 1979; Gaff, 1980), and it has been surmised that recovery is initiated by rehydration-induced repair mechanisms mobilized once full turgor has been achieved (Reynolds and Bewley, 1993). In order to determine the importance of cellular repair during rehydration, and possibly during the drying phase, we chose to analyze the process of protein degradation involving ubiquitin and the 26S proteasome pathway.

Previous work identified ubiquitin as one of a number of proteins possibly involved in the recovery phase of *T. ruralis* and *S. stapfianus* during rehydration (Scott and Oliver, 1994; Oliver *et al.*, 1998). Ubiquitin is a small, highly conserved, 76 amino acid (8.5 kDa) protein found in all eukaryotes either as

a free monomer or covalently linked to a variety of proteins. Though multifunctional (von Kampen *et al.*, 1996), one of its main known functions is in tagging proteins for selective degradation by the 26S proteasome (Hasselgren and Fischer, 1997). Ubiquitin is induced by various stresses in plants and animals (Binet *et al.*, 1991; Christensen *et al.*, 1992; Genschik *et al.*, 1992; Sun and Callis, 1997; Fornace *et al.*, 1998) but desiccation-related effects are poorly understood (Handke *et al.*, 1993; Schulz *et al.*, 1994). Protein degradation is a normal cellular activity but an increase in degradation in response to a stress can be interpreted as the result of excessive protein damage (Ferguson *et al.*, 1990) and an attempt to remove damaged proteins from the cell in order to maintain cellular function. This enables us to use ubiquitin as an indicator of cellular damage/repair in response to desiccation. The fact that ubiquitin expression is sensitive to desiccation in both *S. stapfianus* and *T. ruralis* meant we could also use it to identify the timing of protein damage and thereby directly compare the different systems during drying and rehydration.

In this work we characterize a putative full-length ubiquitin cDNA from *Sporobolus stapfianus*. By comparing the steady-state levels of ubiquitin transcripts and protein profiles from drying and rehydrating tissues of *S. stapfianus* and *Tortula ruralis* we determined the extent and timing of cellular damage/repair in these diverse systems. This allows us to characterize at least one aspect of desiccation-related cellular repair while making a direct comparison of two different desiccation tolerance systems.

## Materials and methods

### *Plant material*

*Sporobolus stapfianus* Gandoger (original provenance Verena, Transvaal, South Africa) and *Sporobolus pyramidalis* Beauv. (northern Cape province) were grown in commercial potting soil in 4.5 liter pots under greenhouse conditions using natural light with an average day/night temperature of 21–27 °C and a relative humidity (RH) of 17%. Plants were watered every morning by misting and individual plants were dried by withholding water. To dry to air dryness (constant weight in ambient conditions) required 15 to 20 days. Rehydration of desiccated plants was achieved by submerging the whole pot and plant in water which contained 100 mg/l carbenicillin (to reduce growth

of soil-borne bacteria which would contaminate leaf samples) for specified time periods. The relative water content (RWC) of a plant was determined from a minimum of three random samples of excised leaf tissue. RWC was calculated by the formula,  $\text{Fwt} - \text{Dwt} / \text{FTwt} - \text{Dwt}$ , where Fwt was the fresh sample weight, FTwt was the full turgor weight of the same sample after submersion in deionized water overnight in the dark, and Dwt was the weight of the sample after being dried at 70 °C overnight (Kuang *et al.*, 1995). Leaf tissue at the required RWC, or following a defined period of rehydration, was used immediately or snap-frozen in liquid nitrogen and stored at -80 °C. Care was taken to collect leaf samples at approximately the same time of day to ensure that changes in transcript levels were not due to the circadian control of gene expression or diurnal effects.

*Tortula ruralis* ([Hedw.] Gaertn, Meyer and Scherb) gametophytes were collected and stored dry at room temperature. For experiments, gametophyte tissue was hydrated for 48 h as described previously (Oliver, 1991; Scott and Oliver, 1994). Rapid-dried moss was prepared by placing fresh gametophyte on filter paper over activated silica gel in a Petri dish as described previously (Bewley and Thorpe, 1974). Slow-dried moss was prepared by placing fresh moss gametophytes of known weights on a nylon mesh in a closed chamber over a stirred saturated solution of sodium nitrite at 20 °C (RH 66%) for 24 h. Using this regime the air-dried weight (ca. 20% of original fresh weight) was obtained within 6 h (Oliver, 1991).

#### Nucleic acid isolation

Total RNA was isolated from fresh or frozen *Sporobolus* leaves and moss gametophyte using a hot phenol method (de Vries *et al.*, 1988). RNA was enriched for poly(A)<sup>+</sup> RNA using oligo-dT-conjugated magnetic beads (Dynal, Oslo, Norway).

Genomic DNA was isolated from 10 g of fresh leaf material by a CTAB-based method (Ausubel *et al.*, 1989). The DNA was purified by ultracentrifugation (Sorvall Ultra 80 ultracentrifuge) on a 6 ml, 1.56 g/ml cesium chloride gradient containing 0.33 µg/ml ethidium bromide and centrifuged at  $315\,000 \times g$  for 6 h in a Sorvall TV 865 rotor. After isolation of the banded DNA, the sample was extracted four times with water-saturated butanol to remove ethidium bromide and the DNA was ethanol-precipitated. DNA and RNA were quantified spectrophotometrically at OD<sub>260 nm</sub>.

#### Ubiquitin cDNA isolation

Total RNA was extracted from a combination of desiccated leaves of *Sporobolus stapfianus* rehydrating for 3, 6 and 12 h, and doubly enriched for poly(A)<sup>+</sup> RNA using oligo-dT magnetic beads (Dynal). A cDNA library with ca.  $2 \times 10^6$  plaque-forming units (p.f.u.) was constructed in the Uni-Zap XR vector using a ZAP cDNA synthesis kit (Stratagene, La Jolla, CA). To identify a putative full-length *Sporobolus stapfianus* ubiquitin cDNA, a partial ubiquitin cDNA clone from the moss *Tortula ruralis* (Scott and Oliver, 1994) was random-prime-labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (DecaPrime II Kit, Ambion) and used to screen ca.  $2 \times 10^5$  p.f.u. in the first screen. Positive plaques were sequentially screened until a homogeneous population of p.f.u. was achieved. The cDNA was rescued from the purified phage isolate as a cDNA fragment in the pBluescript SK- plasmid vector according to the manufacturer's instructions.

#### DNA sequencing

DNA sequencing was performed using dRhodamine and BigDye Terminator Cycle Sequencing kits (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequence reactions were analyzed with a Perkin Elmer/ABI Prism 310 automated sequencer. Primers used were anchored in the pBluescript vector (SK-) Lac I (TTCACACAGGAAACAGCTATGAC), and Lac Z (CGGGCCTCTTCGCTATTAC). Two further primers, Lac I\* (GGAAGCCTTAGCCCATCCTC) in the 5'-untranslated region adjacent to the start codon, and Lac Z\* (CACAGCAGCTCCTGACCAGG) in the 3'-untranslated region, were used to sequence across the coding region.

#### Northern and Southern analysis

Northern blots were prepared by fractionating poly(A)<sup>+</sup>-enriched RNA (1 µg per lane) on a 1.2% formaldehyde-denaturing agarose gel (Ausubel *et al.*, 1989). After rinsing the gel twice in distilled water, RNA was transferred to a Duralon-UV membrane (Stratagene) by pressure-blotting for 1 h at 550 kPa and immobilized on the membrane by UV irradiation using a total of 120 µJ of energy as recommended by the manufacturer (UV Stratalinker 1800, Stratagene).

Southern blots were prepared by fractionating 10 µg samples of genomic DNA on 0.8% agarose gels. DNA was depurinated in the gel by rinsing the

gel in 0.1 M HCl for 30 min, denatured for 30 min in denaturing buffer (1.5 M NaCl, 0.5 M NaOH) and neutralized for 30 min in neutralizing buffer (1.5 M NaCl, 0.5 M Tris-Cl pH 8.0). The gel was rinsed once for 5 min in 0.1 M Tris-Cl pH 8.0, 2× SSC, prior to transfer and immobilization of DNA to a Duralon-UV membrane by the same procedure described for northern blotting.

Membranes were incubated overnight at 62 °C in prehybridization buffer (0.5 M sodium phosphate pH 7.1, 1% SDS, 1% BSA, 100 µg/ml of sonicated salmon sperm DNA). Nucleic acid probes were random-prime-labeled (Decaprime II Kit; Ambion, Austin, TX) with [ $\alpha$ -<sup>32</sup>P]dCTP (NEN-Dupont) and unincorporated label removed by purification columns (Stratagene). Probes were incubated at 95 °C for 5 min, added directly to the prehybridization solution and allowed to hybridize overnight at 62 °C. Membranes were washed initially at 42 °C and as high as 62 °C in wash buffer (40 mM sodium phosphate pH 7.1, 0.1% SDS) until the membrane registered near background readings on a hand-held monitor.

#### Western analysis

Protein was extracted from whole *S. stapfianus* leaves and *T. ruralis* gametophyte at specific RWC values or at certain time points during drying and rehydration. Leaf tissue was excised, snap-frozen in liquid nitrogen and ground to a fine powder in a pre-chilled mortar and pestle. The powder (1 g) was mixed with 5 ml of chilled extraction buffer (0.5 M Tris-HCl pH 8.65, 50 mM EDTA, 0.1 M KCl, 2% 2-mercaptoethanol) to give a fluid consistency. After 5 min on ice, with regular agitation, cell debris and insoluble material were removed by centrifugation twice for 10 min at 14 000 × *g* each. Proteins were extracted from the aqueous phase in an equal volume of water-saturated phenol, and the phenol phase re-extracted with wash buffer (extraction buffer without 2-mercaptoethanol). Proteins were precipitated from the phenol phase by addition of 2.5 volumes of 0.1 M ammonium acetate in methanol and incubation at −20 °C for at least 1 h. Precipitated proteins were recovered by centrifugation, washed twice in 0.1 M ammonium acetate in methanol and allowed to air-dry. After resuspension in wash buffer, protein concentrations were estimated by the BioRad protein assay (BioRad, Hercules, CA) with BSA as standard. Proteins were fractionated by 12% SDS-PAGE using the BioRad minigel system and transferred to PVDF membrane (Pierce, Rock-

ford, IL) using the BioRad TransBlot in transfer buffer (5.8 g/l Tris base, 2.9 g/l glycine and 200 ml/l 100% methanol).

Membranes were blocked for 1 h in TBS containing 0.1% Tween 20 (TTBS) and 5% non-fat dried milk. Ubiquitin antibody developed in rabbit (Sigma, St. Louis, MO) at 1:100 dilution was incubated with the membrane in TTBS for 1 h. After 3 × 5 min washes in TTBS, goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Pierce) was incubated with the membrane at a dilution of 1:10 000 in TTBS for 1 h. After 3 × 5 min washes in TTBS, signal detection was achieved with the SuperSignal Substrate system (Pierce).

#### ABA treatment

Whole leaves of *S. stapfianus* were excised from the plant and placed in water containing a 100 µM racemic mix of abscisic acid (Calbiochem-Behring) or just water as control. A fresh cut of the base of the leaves was made under water to ensure a continuous water column within each leaf. Leaves were harvested at 2, 4 and 6 h for the ABA treatment, and 2 h for the water control.

## Results

#### Ubiquitin cDNA isolation and characterization

A putative full-length ubiquitin cDNA of 1423 bp (GenBank accession number AF148448) was isolated from a *Sporobolus stapfianus* rehydration leaf cDNA library using poly(A)<sup>+</sup> RNA pooled from a combination of desiccated leaves rehydrating for 3, 6 and 12 h. The cDNA has a putative open reading frame (ORF) of 1143 bp starting at nucleotide 66 that is flanked by a 65 bp 5' non-coding region and a 3' non-coding region of 215 bp that includes 20 bp of poly(A)<sup>+</sup> tail. The putative ORF encodes a polypeptide of 381 amino acids that consists of five head-to-tail ubiquitin monomer coding sequences. Translation of the ORF would theoretically result in the production of four monomers of 76 amino acids in length (monomers 1 to 4) and one monomer of 77 amino acids. The fifth monomer has an extra amino acid at its carboxy-terminus, a glutamine residue, similar to the terminal monomer sequence reported for a maize polyubiquitin (Christensen *et al.*, 1992).

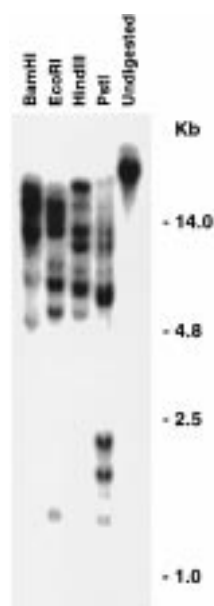


Figure 1. Southern analysis of genomic DNA from *Sporobolus stapfianus*. Genomic DNA was isolated from *S. stapfianus* and cesium chloride-purified. Undigested DNA and DNA digested with *Bam*HI, *Eco*RI, *Hind*III and *Pst*I (10  $\mu$ g per lane) was separated on a 0.8% agarose gel, transferred to Duralon-UV membrane and probed with the full *S. stapfianus* ubiquitin cDNA.

#### Southern analysis

Genomic DNA from *S. stapfianus* was digested with four restriction enzymes that did not have predicted restriction sites within the ubiquitin cDNA: *Bam*HI, *Eco*RI, *Hind*III and *Pst*I. Southern analysis of this DNA, with the full-length *S. stapfianus* ubiquitin cDNA as a probe, revealed several strongly hybridizing bands in each of the four genomic DNA restriction enzyme digests (Figure 1). In addition to the major hybridization bands, each digest revealed multiple bands that weakly hybridize to the cDNA probe. The complex hybridization patterns seen in each digest indicate that ubiquitin is not encoded by a single gene at a single chromosomal location in *S. stapfianus*.

#### Ubiquitin transcript modulation during drying and rehydration in *Sporobolus* and *Tortula* ruralis

In order to examine the changes in ubiquitin transcript levels during desiccation and rehydration, northern blots of poly(A)<sup>+</sup>-enriched RNA from leaf tissues of *S. stapfianus*, *S. pyramidalis* and gametophyte of *T. ruralis* were examined using the full *S. stapfianus* ubiquitin cDNA as a probe (Figures 2 and 3). The *S. stapfianus* ubiquitin cDNA was used as a probe for

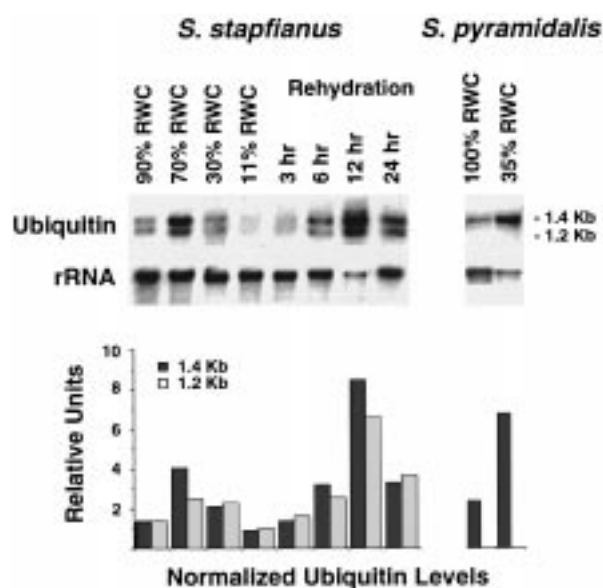


Figure 2. Northern analysis of poly(A)<sup>+</sup> RNA from *Sporobolus stapfianus* and *Sporobolus pyramidalis* during drying and rehydration of leaves. Total RNA was isolated from leaves and enriched for poly(A)<sup>+</sup> RNA. Poly(A)<sup>+</sup> RNA (1  $\mu$ g per lane) was separated on a 1.2% formaldehyde denaturing agarose gel, transferred onto Duralon UV membrane and probed with the full *S. stapfianus* ubiquitin cDNA. As a control of lane loading the blot was stripped and reprobed with a sorghum rDNA probe to 18S RNA (Wood and Goldsborough, 1997). RNA extracted from leaves during dehydration is represented by relative water content (%RWC) while RNA isolated during rehydration of desiccated leaves is represented by hours of rehydration.

*Sporobolus* and *Tortula* RNAs both for convenience and to maximize signal strength. Both the *Tortula* and *Sporobolus* ubiquitin cDNAs hybridized to all of the ubiquitin transcripts within the RNA from either plant (data not shown) but the *Sporobolus* polyubiquitin cDNA has five copies of the ubiquitin monomer in contrast to the partial *Tortula* polyubiquitin cDNA which encodes only two.

In *S. stapfianus* two transcripts hybridized to the polyubiquitin probe, one of ca. 1.4 kb and one of ca. 1.2 kb. Both of these ubiquitin transcripts accumulated during the early stages of drying, with a peak in accumulation at a relative water content (RWC) between 77% and 30%, but decreased to very low levels in fully dried tissue. The ubiquitin transcripts accumulated more during rehydration than drying, reaching peak levels at around 12 h, before returning to near control levels by 24 h. Data shown in Figure 3 indicate that the 1.4 kb transcript accumulated to a greater degree during drying and rehydration than the 1.2 kb transcript. Northern analysis of the desiccation-sensitive

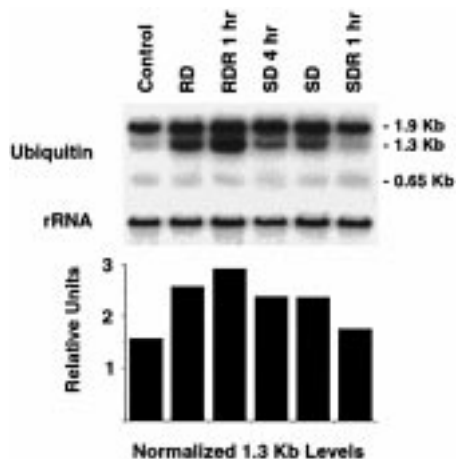


Figure 3. Northern analysis of poly(A)<sup>+</sup> RNA from the moss *Tortula ruralis* during drying and rehydration. Total RNA was isolated from *T. ruralis* gametophyte and enriched for poly(A)<sup>+</sup> RNA. Poly(A)<sup>+</sup> RNA was separated on a 1.2% formaldehyde denaturing agarose gel (1  $\mu$ g per lane), transferred onto Duralon UV membrane and probed with the full *S. stapfianus* ubiquitin cDNA. As a control of lane loading the blot was stripped and re-probed with a sorghum rDNA probe to 18S RNA (Wood and Goldsborough, 1997). Moss was rapid-dried (RD) over activated silica gel and subsequently rehydrated (RDR), and slow-dried (SD) as previously described (Oliver, 1991), and subsequently rehydrated (SDR).

grass *S. pyramidalis* (Figure 2) revealed only one ubiquitin transcript of 1.4 kb present in both untreated and drying leaves. This transcript also accumulated during drying, similar to that for *S. stapfianus*, but as the tissues of this plant are destroyed upon rehydration we were unable to examine this part of the wet-dry cycle.

Northern analysis of poly(A)<sup>+</sup> RNA from drying and rehydrating gametophytes of *Tortula ruralis* (Figure 4) revealed the presence of three ubiquitin transcripts of 0.65 kb, 1.3 kb and 1.9 kb. Steady-state levels of the 0.65 kb and the 1.9 kb transcripts were relatively unaffected by either drying or rehydration. However, while the levels of the 1.3 kb transcript were similar in rapid- or slow-dried tissue, accumulation continued in the first hour of rehydration of rapid-dried tissue but declined in slow-dried tissue.

#### Response of *S. stapfianus* ubiquitin transcript levels to exogenous ABA

Many plant desiccation-responsive genes are also induced by exogenous ABA (Ingram and Bartels, 1996). We were interested in determining whether exogenous ABA could act as a signal transducer and result in the accumulation of ubiquitin transcripts in *S. stapfianus*. Earlier work (M.J. Oliver, unpublished data) demon-



Figure 4. Northern analysis of poly(A)<sup>+</sup> RNA from *Sporobolus stapfianus* control leaves and leaves treated with ABA. RNA was isolated from *S. stapfianus* leaves standing in water for 2 h as control, or in water containing a 100  $\mu$ M racemic mix of abscisic acid for 2, 4 and 6 h. Poly(A)<sup>+</sup> RNA (1  $\mu$ g per lane) was separated on a 1.2% formaldehyde denaturing agarose gel, transferred onto Duralon UV membrane and probed with the full *S. stapfianus* cDNA. To show that the exogenous ABA was capable of effecting transcript accumulation, the blot was stripped and re-probed with a partial cDNA of a sorghum dehydrin (a gift from Dr A.J. Wood, Southern Illinois University, Carbondale). As a control of lane loading the blot was stripped and re-probed with a sorghum rDNA to 18S RNA (Wood and Goldsborough, 1997).

strated that ABA is undetectable in *Tortula* tissues and is not involved in the response to desiccation. Northern blots of poly(A)<sup>+</sup> RNA extracted from *S. stapfianus* leaves standing in ABA solution and leaves standing in water alone as control were probed with the full ubiquitin cDNA. None of the transcript levels were altered appreciably in response to the exogenous ABA (Figure 4) suggesting that ABA does not influence ubiquitin accumulation in *S. stapfianus*. To ensure that exogenous ABA was influencing transcript levels in the grass leaves, the same blot was stripped and re-probed with a PCR fragment coding for part of an ABA-inducible dehydrin cDNA from sorghum (a gift from Dr A.J. Wood, Southern Illinois University, Carbondale). This blot demonstrates an increase in transcript levels of a corresponding *Sporobolus* dehydrin transcript within 2 h of exposure to ABA and which continued to increase throughout the 6 h of ABA application. To ensure lanes contained similar amounts of RNA, the blot was stripped and re-probed with an rDNA probe (Wood and Goldsborough, 1997).

#### Western analysis of *S. stapfianus* and *Tortula ruralis* ubiquitin during drying and rehydration

Western analysis with commercial ubiquitin antiserum indicated that leaves of *S. stapfianus* contained both free and conjugated ubiquitin in fully hydrated (control), as well as drying, dried and rehydrating leaves (Figure 5). Free monomer, dimers and trimers of ubiquitin

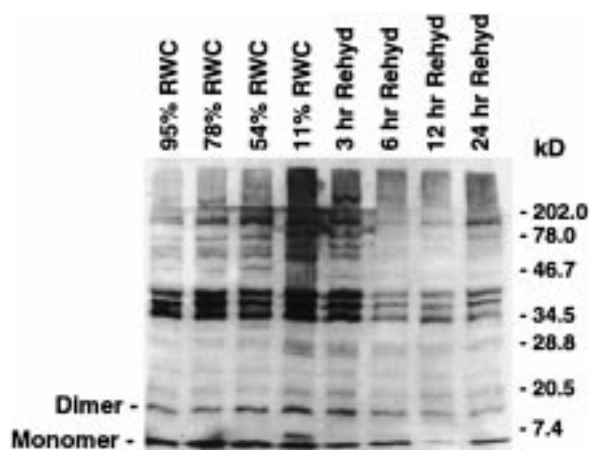


Figure 5. Western analysis of proteins from *Sporobolus stapfianus* during drying and rehydration. Total proteins were extracted from leaves of *S. stapfianus*, separated by 12% SDS PAGE (10  $\mu$ g per lane) and transferred onto PVDF membrane. Blots were incubated with a commercially available primary antibody to ubiquitin (1:100) followed by a secondary HRP-conjugated goat anti-rabbit antibody (1:10 000). Signal was detected using the Pierce Supersignal chemiluminescent system. Lanes of protein isolated during dehydration are represented as relative water content (% RWC) while proteins isolated during rehydration are listed as hours of rehydration.

ubiquitin as well as conjugated ubiquitin were evident in all samples. Monomer levels increased slightly during drying, remained high in dried leaves and, except for a marked reduction in the 12 h sample, maintained constant, but lower, levels throughout rehydration. High-molecular-weight conjugates representing proteins tagged for removal appeared to increase during drying and to decrease rapidly upon rehydration. While several bands appeared to vary in intensity during drying and to rehydration one band, slightly larger than the monomer, was detected in dried grass but was barely detectable in any other sample.

Ubiquitin protein levels in *Tortula ruralis* were analyzed with the same polyclonal antiserum as for *S. stapfianus* (Figure 6). Ubiquitin monomer levels were slightly elevated relative to control levels in all samples except for slow-drying tissue. This slow-drying gametophyte was the only tissue to contain significant levels of high-molecular-weight conjugated ubiquitin.

## Discussion

We have isolated and characterized a polyubiquitin cDNA encoding five head-to-tail ubiquitin monomers from the resurrection grass *Sporobolus stapfianus*. In

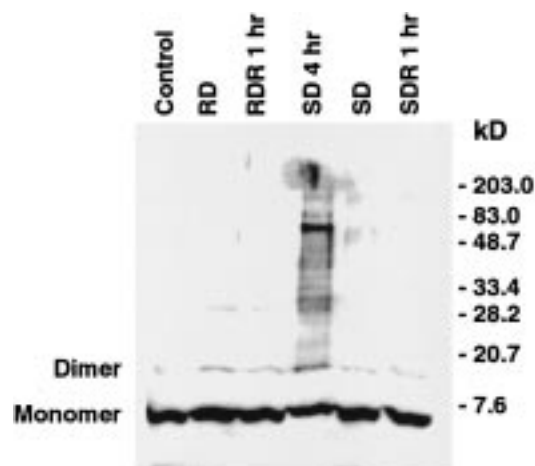


Figure 6. Western analysis of proteins from the moss *Tortula ruralis* during drying and rehydration. Total proteins were extracted from gametophyte tissue of *T. ruralis*, separated by 12% SDS PAGE (10  $\mu$ g per lane) and transferred onto PVDF membrane. Blots were incubated with a primary commercial antibody to ubiquitin (1:100) followed by a secondary HRP-conjugated goat anti-rabbit antibody (1:10 000). Signal was detected using the Pierce Supersignal chemiluminescent system. Tissue was rapid-dried over activated silica gel (RD) and subsequently rehydrated (RDR) and slow-dried (SD) and subsequently rehydrated (SDR) for 1 h.

some plants such as *Arabidopsis* (Burke *et al.*, 1998) and sunflower (Binet *et al.*, 1991) ubiquitin is encoded by up to 10 genes, and our results indicate that *S. stapfianus* ubiquitin is also encoded by a multi-gene family. We have utilized this cDNA to investigate the response of ubiquitin genes to desiccation and rehydration of vegetative tissues of desiccation-tolerant plants, in particular the modified desiccation-tolerant South African grass *Sporobolus stapfianus* and the fully desiccation-tolerant moss *Tortula ruralis*.

Ubiquitin acts as a tag for the selective degradation of short-lived, denatured, incomplete or misfolded proteins via the 26S proteasome pathway (von Kampen *et al.*, 1996; Hasselgren and Fischer, 1997). Various types of stress have been shown to increase ubiquitin transcript levels in mammalian cells (Fornace *et al.*, 1989), yeast (Finley *et al.*, 1987), *Chlamydomonas* (Ligr and Malek, 1997) and plants (Ferguson *et al.*, 1990; Binet *et al.*, 1991; Christensen *et al.*, 1992; Genschik *et al.*, 1992; Sun and Callis, 1997). The response of plant ubiquitin to stresses associated with temperature fluctuation, various chemicals and viral or bacterial infections are well documented but little has been reported on ubiquitin involvement in response to water stress (Handke *et al.*, 1993; Schulz *et al.*, 1994). Results presented here (Figures 2 and 3)

demonstrate that certain ubiquitin transcript levels are modulated in response to dehydration and rehydration in at least some modified and fully desiccation-tolerant plants, as well as a desiccation-sensitive plant.

Several ubiquitin transcripts of various size have been identified in plants, with possible individual roles including housekeeping, developmental and cell cycle regulation and stress responses (Genschik *et al.*, 1992). In *S. stapfianus* two ubiquitin transcripts of 1.4 and 1.2 kb were evident in all leaf samples assayed, both accumulating during desiccation but to greater levels during the first 12 h of rehydration. By 24 h of rehydration both ubiquitin transcripts had returned to near control levels. Western analysis revealed greater levels of ubiquitin-tagged proteins in drying and dried tissue. This increase in tagged proteins was possibly the result of lower proteolysis rates by the 26S proteasome due to water depletion as these levels were reduced within 6 h of rehydration at which time *S. stapfianus* leaves have reached control levels of RWC (P. O'Mahony and M. Oliver, unpublished data). However, changes in the levels of ubiquitin-conjugated proteins can be a reflection of altered rates of ubiquitination, deubiquitination or proteolysis by the 26S proteasome and thus further analysis is required before such changes can be explained. The greater accumulation of transcripts during rehydration and the reduction in the levels of ubiquitin monomer 12 h into the rehydration episode suggests a greater degree of damage incurred by rehydration. Previous work on *S. stapfianus* described Rab2, an endomembrane protein involved in vesicular trafficking, that has transcript levels augmented during drying and in the dried state as well as 12 h after initiation of rehydration (O'Mahony and Oliver, 1999). However, contrary to ubiquitin transcripts, the increase in Rab2 transcript after 12 h of rehydration was considerably less than that during dehydration. In addition, Rab2 transcripts returned to control levels by 24 h of rehydration whereas ubiquitin transcripts were still elevated at that time. Other work involving the use of differential display to identify genes important to desiccation tolerance in *S. stapfianus* identified a number of transcripts whose levels were not specifically altered 12 h after rehydration (P. O'Mahony and M. Oliver, unpublished data). These studies also revealed a chloroplast rRNA which suffers degradation only during rehydration but primarily 12 h after initiation of re-watering. These data suggest that 12 h of rehydration is a critical time point in the recovery of *S. stapfianus* where new and more intense protein degradation occurs. This

idea is supported by earlier observations that rehydration rather than desiccation results in the greatest membrane damage (Oliver *et al.*, 1997), and since water stress has been shown to correlate to increased levels of membrane-associated ubiquitin conjugates (Schulz *et al.*, 1994), it is feasible that much of the rehydration-associated damage is due to an injurious swelling effect on membranes. Furthermore, physiological studies of drying and rehydrating *S. stapfianus* demonstrated that photosynthetic activity and CO<sub>2</sub> assimilation require several days of rehydration before recovering (Di Blasi *et al.*, 1998) suggesting that the relatively early peak in ubiquitin activity during rehydration is associated with the recovery process.

The phytohormone abscisic acid (ABA) is known to be a mediator of desiccation tolerance in a number of resurrection plants (Ingram and Bartels, 1996) and some, but not all, desiccation-induced proteins are also inducible by exogenous ABA. Though a number of genes are inducible by ABA and water deficit in *S. stapfianus* (Blomstedt *et al.*, 1998), desiccation tolerance is independent of ABA because tolerance is induced as the plant dries to 60% RWC while endogenous ABA levels rise later in drying (Gaff and Loveys, 1993). Our results demonstrate that *S. stapfianus* ubiquitin transcripts are non-responsive to exogenous ABA. In addition, it appears that ubiquitin transcripts accumulate early in response to moderate drought stress (90–70% RWC) coinciding with the first phase of novel protein synthesis in *S. stapfianus* and preceding the rise in endogenous ABA (Gaff and Loveys, 1993; Kuang *et al.*, 1995). This observation is consistent with earlier reports that to date ubiquitin genes have been observed to respond only to the plant hormone kinetin (Muhitch and Shatters, 1998).

*Tortula ruralis* is fully desiccation-tolerant, which means it can withstand even rapid drying of its vegetative tissues. This study has identified three *Tortula* ubiquitin transcripts, of 0.65 1.3 and 1.9 kb, that were present under all conditions. The 0.65 kb transcript, because of its size, probably encodes a ubiquitin monomer fused at the 3' end to an extension protein, usually a ribosomal protein (Callis *et al.*, 1990) and, like the 1.9 kb transcript, remained at constant levels throughout drying and rehydration. The 1.3 kb message accumulated in response to both rapid and slow drying, and also to rehydration, but only if the tissue was dried rapidly. This result is consistent with previous results (Scott and Oliver, 1994) which described a ubiquitin transcript that accumulated in response to rehydration of rapid-dried gametophyte. However,

the accumulation of the 1.3 kb ubiquitin transcript during rapid desiccation is in contrast to other rehydrins whose transcripts accumulate during slow but not rapid drying (M.J. Oliver, unpublished data; Oliver and Wood, 1997). It appears that all *Tortula* ubiquitin transcripts are stable during desiccation and in the dried state, unlike ubiquitin transcripts in *S. stapfianus* which are depleted in dried tissue. Accumulation of the 1.3 kb transcript during drying may indicate that there is an increase in the stability of this mRNA, or that some transcription is still possible even under water stress. Recent work (Wood and Oliver, 1999) demonstrated the formation of messenger ribonucleoprotein particles (mRNPs) during slow desiccation which may be linked to the accumulation of rehydrins. Though formation of mRNPs during rapid drying has not been observed, it is possible that accumulation of the 1.3 kb ubiquitin transcript is a result of similar sequestration. The observation that rapid-dried gametophytes continue to accumulate the 1.3 kb transcript upon rehydration is consistent with a large body of evidence that rapid desiccation of *Tortula* gametophytes results in greater cellular damage than slow drying, thereby necessitating more repair (Bewley and Oliver, 1992). It is notable that in the modified desiccation-tolerant plant *S. stapfianus*, ubiquitin transcripts are present in much reduced levels in dried tissue. These plants dry slowly, building up cellular protection during drying, and also probably undergo less cellular damage than if dried rapidly. This would then allow ubiquitin protein, stored during desiccation, to tag proteins damaged late in dehydration and early in rehydration while transcript levels are replenished.

Western analysis revealed high-molecular-weight ubiquitin conjugates in *S. stapfianus* leaves under all conditions in contrast to *I. ruralis* which displayed significant levels of these conjugates only in slow-drying tissue. Since the level of ubiquitinated proteins can be dependent on the rates of ubiquitination, deubiquitination or degradation by the 26S proteasome it would be speculative to explain the difference in ubiquitination patterns between *S. stapfianus* and *T. ruralis*. However, a reduced requirement for inducible ubiquitin transcript in rehydrating slow-dried *Tortula*, coupled to the appearance of conjugated ubiquitin during slow drying, may suggest that removal of some dehydration-damaged proteins during desiccation may help to facilitate faster recovery of slow-dried tissue.

We have shown that both the modified and fully desiccation-tolerant plants analyzed in this study accumulate ubiquitin transcripts in response to drying

and rehydration of their vegetative tissue. This reflects an increase in requirement for ubiquitin either to replenish depleted stocks or in response to an increase in protein degradation via the ubiquitin-mediated 26S proteasome pathway. Ubiquitin is considered a stress protein and thus its response to water loss in plants may be a general stress response, an idea supported by the induction of a ubiquitin transcript during drying of the desiccation-sensitive *S. pyramidalis*. However, the presence of multiple ubiquitin transcripts, constitutive and inducible, in drying and rehydrating *S. stapfianus* and *T. ruralis* suggests that the availability of sufficient ubiquitin monomer in times of critical cell stress may be important for survival of these plants. In tomato, the stress-related induction of a ubiquitin-conjugating enzyme (Feussner *et al.*, 1997) suggests that sufficient ubiquitin monomer is provided by a plant at critical periods whether by recycling of current stocks or by synthesis of nascent monomer. In the case of drying and rehydrating *S. stapfianus*, sufficient ubiquitin is provided by induced ubiquitin, and in *T. ruralis* by induced and constitutive ubiquitin. The ability of *T. ruralis* to accumulate and store ubiquitin transcripts, even during rapid desiccation when overall mRNA levels decline (Oliver and Bewley, 1984a,b), suggests a strategic role in its rapid recovery from desiccation. Though cell protection mechanisms are considered to play a major role in desiccation tolerance, this work indicates that the role of repair mechanisms, such as that represented by ubiquitin-mediated protein degradation may be more than supplemental. Still further characterization of the ubiquitin-mediated 26S proteasome pathway, as well as other facets of the repair machinery, are required to fully appreciate the role of damage and repair in desiccation tolerance in plants.

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## References

- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. 1989. Current Protocols in Molecular Biology. Green/Wiley Interscience, New York.
- Bartels, D.R., Alexander, K., Schneider, R., Elster, R., Velasco, J., Alamillo, G., Bianchi, G., Nelson, D. and Salamini, F. 1993. Desiccation-related gene products analyzed in a resurrection plant and in barley embryos. In: Close, T.J. and Bray, E.A. (Eds.), Plant Responses to Cellular Dehydration during Environmental Stress. Current Topics in Plant Physiology, Series 10, American Society of Plant Physiologists, pp. 119–127.
- Bernacchia, G., Salamini, F. and Bartels, D. 1996. Molecular characterization of the rehydration process in the resurrection plant *Craterostigma plantagineum*. Plant Physiol. 111: 1043–1050.
- Bewley, J.D. 1979. Physiological aspects of desiccation-tolerance. Annu. Rev. Plant Physiol. 30: 195–238.
- Bewley, J.D. and Krochko, J.E. 1982. Desiccation-tolerance. In: Lange, O.L., Nobel, P.S., Osmond, C.B. and Ziegler H. (Eds.), Encyclopedia of Plant Physiology, vol. 12B. Physiological Ecology II, Springer-Verlag, Berlin, pp. 325–378.
- Bewley, J.D. and Oliver, M.J. 1992. Desiccation-tolerance in vegetative plant tissues and seeds: protein synthesis in relation to desiccation and a potential role for protection and repair mechanisms. In: Osmond, C.B. and Somero, G. (Eds.), Water and Life: A Comparative Analysis of Water Relationships at the Organismic, Cellular and Molecular Levels, Springer-Verlag, Berlin, pp. 141–160.
- Bewley, D. and Thorpe, T. 1974. On the metabolism of *Tortula ruralis* following desiccation and freezing: respiration and carbohydrate oxidation. Physiol. Plant. 32: 147–153.
- Bianchi, G., Gamba, A., Murelli, C., Salamini, F. and Bartels, D. 1992. Low molecular weight solutes in desiccated and ABA-treated calli and leaves of *Craterostigma plantagineum*. Phytochemistry 31: 1917–1922.
- Binet, M.-N., Weil, J.-H. and Tessier, L.-H. 1991. Structure and expression of sunflower ubiquitin genes. Plant Mol. Biol. 17: 395–407.
- Blomstedt, C.K., Neale, A.D., Gianello, R.D., Hamill, J.D. and Gaff, D.F. 1998. Differential gene expression in desiccation-tolerant and desiccation-sensitive tissue of the resurrection grass, *Sporobolus stapfianus*. Aust. J. Plant Physiol. 25: 937–946.
- Burke, T., Callis, J. and Vierstra, R. 1988. Characterization of a polyubiquitin gene from *Arabidopsis thaliana*. Mol. Gen. Genet. 213: 435–443.
- Callis, J., Raasch, J. and Vierstra, R. 1990. Ubiquitin extension proteins of *Arabidopsis thaliana*. Structure, localization, and expression of their promoters in transgenic tobacco. J. Biol. Chem. 265: 12486–12493.
- Christensen, A., Sharrock, R. and Quail, P. 1992. Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol. Biol. 18: 675–689.
- Close, T.J. 1996. Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. Physiol. Plant. 97: 795–803.
- de Vries, S., Hoge, H. and Bisseling, T. 1988. Isolation of total and polysomal RNA from plant tissues. In: Gelvin, S.B. and Schilperoort, R.A. (Eds.), Plant Molecular Biology Manual, Kluwer Academic Publishers, Dordrecht, Netherlands pp. B6: 1–13.
- Di Blasi, S., Puliga, S., Losi, L. and Vazzana, C. 1998. *S. stapfianus* and *E. curvula* cv. Consol in vivo photosynthesis, PSII activity and ABA content during dehydration. Plant Growth Regul. 25: 97–104.
- Ferguson, D., Guikema, J. and Paulsen, G. 1990. Ubiquitin pool modulation and protein degradation in wheat roots during high temperature stress. Plant Physiol. 92: 740–746.
- Feussner, K., Feussner, I., Leopold, I. and Wasternack, C. 1997. Isolation of a cDNA for an ubiquitin-conjugating enzyme UBC1 of tomato: the first stress-induced UBC of higher plants. FEBS Lett. 409: 211–215.
- Finley, D., Ozkaynak, E. and Varshavsky, A. 1987. The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation and other stresses. Cell 48: 1035–1046.
- Fornace, A., Alamo, I., Hollander, C. and Lamoreaux, E. 1989. Ubiquitin mRNA is a major stress induced transcript in mammalian cells. Nucl. Acids Res. 17: 1215–1230.
- Furini, A., Parcy, F., Salamini, F. and Bartels, D. 1996. Differential regulation of two ABA-inducible genes from *Craterostigma plantagineum* in transgenic *Arabidopsis* plants. Plant Mol. Biol. 30: 343–349.
- Gaff, D.F. and Loveys, B.R. 1993. Absciscic acid levels in drying plants of a resurrection grass. Transact. Malaysian Soc. Plant Physiol. 3: 286–287.
- Gaff, D. 1980. Protoplasmic tolerance to extreme water stress. In: Turner, N.C. and Kramer, P.J. (Eds.), Adaptation of Plants to Water and High Temperature Stress, Wiley Interscience, New York, pp. 207–230.
- Genschik, P., Parmentier, Y., Durr, A., Marbach, J., Criqui, M.-C., Jamet, E. and Fleck, J. 1992. Ubiquitin genes are differentially regulated in protoplast-derived cultures of *Nicotiana sylvestris* and in response to various stresses. Plant Mol. Biol. 20: 897–910.
- Handke, C., Boyle, C. and Wettern, M. 1993. Effect of aging, abiotic and biotic stress upon ubiquitination in young barley plants. Angew. Bot. 67: 120–123.
- Hasselgren, P.-O. and Fischer, J. 1997. The ubiquitin-proteasome pathway: review of a novel intracellular mechanism of muscle protein breakdown during sepsis and other catabolic conditions. Ann. Surg. 225: 307–316.
- Ingram, J. and Bartels, D. 1996. The molecular basis of dehydration tolerance in plants. Annu. Rev. Plant Mol. Biol. 47: 377–403.
- Kaiser, K., Gaff, D. and Whitelaw, W. 1985. Sugar content of leaves of desiccation-sensitive and desiccation-tolerant plants. Naturwissenschaften 72: 608–609.
- Kuang, J., Gaff, D.F., Gianello, R.D., Blomstedt, C.K., Neale, A.D. and Hamill, J.D. 1995. Changes in *in vivo* protein complements in drying leaves of the desiccation-tolerant grass *Sporobolus stapfianus* and the desiccation-sensitive grass *Sporobolus pyramidalis*. Aust. J. Plant Physiol. 22: 1027–1034.
- Ligr, M. and Malek, L. 1997. Ubiquitin metabolism in *Chlamydomonas reinhardtii* following cold shock. Physiol. Plant. 101: 865–871.
- Muhitch, M. and Shatters, R. 1998. Regulation of the maize ubiquitin (Ubi-1) promoter in developing maize (*Zea mays* L.) seeds examined using transient gene expression in kernels grown *in vitro*. Plant Cell Rep. 17: 476–481.
- Oliver, M.J. 1991. Influence of protoplasmic water loss on the control of protein synthesis in the desiccation-tolerant moss *Tortula ruralis*. Plant Physiol. 97: 1501–1511.
- Oliver, M. and Bewley, D. 1984a. Plant desiccation and protein synthesis V: Stability of poly(A)<sup>−</sup> and poly(A)<sup>+</sup> RNA during desiccation and their synthesis upon rehydration in the desiccation-tolerant moss *Tortula ruralis* and the intolerant moss *Cratoneuron filicinum*. Plant Physiol. 74: 917–922.
- Oliver, M. and Bewley, D. 1984b. Plant desiccation and protein synthesis IV: RNA synthesis, stability and recruitment of RNA into protein synthesis upon rehydration of the desiccation-tolerant moss *Tortula ruralis*. Plant Physiol. 74: 21–25.

- Oliver, M.J. and Bewley, J.D. 1997. Desiccation-tolerance of plant tissues: a mechanistic overview. *Hort. Rev.* 18: 171–213.
- Oliver, M.J. and Wood, A.J. 1997. Desiccation tolerance in mosses. In: Koval (Ed.) *Stress Inducible Processes in Higher Eukaryotic Cells*, Plenum Press, New York, pp. 1–25.
- Oliver, M.J., Wood, A.J. and O'Mahony, P.J. 1997. How some plants recover from vegetative desiccation: a repair based strategy. *Acta Physiol. Plant.* 19: 419–425.
- Oliver, M.J., Wood, A.J. and O'Mahony, P.J. 1998. 'To Dryness and Beyond': preparation for the dried state and rehydration in vegetative desiccation-tolerant plants. *Plant Growth Regul.* 24: 193–201.
- O'Mahony, P.J. and Oliver, M.J. 1999. Characterization of a desiccation-responsive small GTP-binding protein (Rab2) from the desiccation-tolerant grass *Sporobolus stapfianus*. *Plant Mol. Biol.* 39: 809–821.
- Reynolds, T. and Bewley, D. 1993. Characterization of protein synthetic changes in a desiccation-tolerant fern *Polypodium virginianum*. Comparison of the effects of drying, rehydration and abscisic acid. *J. Exp. Bot.* 44: 921–928.
- Schneider, K., Wells, B., Schmelzer, E., Salamini, F. and Bartels, D. 1993. Desiccation leads to the rapid accumulation of both cytosolic and chloroplastic proteins in the resurrection plant *Craterostigma plantagineum* Hochst. *Planta* 189: 120–131.
- Schulz, M., Janben, M., Knop, K. and Schnabl, H. 1994. Stress and age related spots with immunoreactivity to ubiquitin-antibody at protoplast surfaces. *Plant Cell Physiol.* 35: 551–556.
- Schwab, K.B. and Gaff, D.F. 1990. Influence of compatible solutes on soluble enzymes from desiccation-tolerant *Sporobolus stapfianus* and desiccation-sensitive *Sporobolus pyramidalis*. *J. Plant Physiol.* 137: 208–215.
- Scott, H. and Oliver, M. 1994. Accumulation and polysomal recruitment of transcripts in response to desiccation and rehydration of the moss *Tortula ruralis*. *J. Exp. Bot.* 45: 577–583.
- Sun C-W. and Callis, J. 1997. Independent modulation of *Arabidopsis thaliana* polyubiquitin mRNAs in different organs and in response to environmental changes. *Plant J.* 11: 1017–1027.
- von Kampen, J., Wetter, M. and Schulz, M. 1996. The ubiquitin system in plants. *Physiol. Plant.* 97: 618–624.
- Wood, A.J. and Goldsborough, P.B. 1997. Characterization and expression of dehydrins in water-stressed *Sorghum bicolor*. *Physiol. Plant.* 99: 144–152.
- Wood, A.J. and Oliver, M.J. 1999. Translational control in plant stress: the formation of messenger ribonucleoprotein particles (mRNPs) in response to desiccation of *Tortula ruralis* gametophytes. *Plant J.* 18: 359–371.